JOURNAL OF BACTERIOLOGY, July 1968, p. 93-97
Vol. 96, No. 1


# Purification and Properties of Peroxidase from *Nitrosomonas europaea*[1]

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Received for publication 20 April 1968

Peroxidase from the obligate chemosynthetic bacterium *Nitrosomonas europaea* was purified 1,500-fold, and its properties were examined. The enzyme had a molecular weight of 53,000 and exhibited characteristic absorption maxima at 410, 524, and 558 mμ. The optimal *p*H and temperature were 7.5 and 44 C, respectively. The peroxidase reaction had an energy of activation of 5,850 cal/mole and required a primary substrate ($H_2O_2$) concentration of $7 \times 10^{-6}$ M to proceed at half maximal velocity ($K_m$). Reduced cytochrome, *c*, *p*-phenylenediamine and pyrogallol acted as hydrogen donors to the purified peroxidase-$H_2O_2$ complex. Conditions most suitable for the chemical oxidation of ammonium by $H_2O_2$ were determined. The reaction was rapid and produced nitrite but no nitrate. Hydroxylamine was not detected as an intermediate, but it could substitute for ammonium in the system. Neither the rate nor the extent of these reactions was influenced by purified peroxidase, and no evidence was obtained to support a conclusion that the enzyme performs a vital role in the transformation of ammonium to nitrite by *N. europaea*.

*Nitrosomonas europaea* is an obligate chemosynthetic bacterium that oxidizes ammonium ($NH_4^+$) to nitrite ($NO_2^-$) via a pathway that includes hydroxylamine ($NH_2OH$) as an intermediate (18). The conversion of $NH_2OH$ to $NO_2^-$ has been performed with cell-free systems that are particulate and contain flavine, cytochromes *a*, *b*, and *c*, and $NH_2OH$-cytochrome *c* reductase (12, 15). However, in one report (29), the terminal oxidase is described as soluble rather than particulate and of the cytochrome *o* rather than of the cytochrome *a* type. More is known of the oxidation of $NH_2OH$ to $NO_2^-$ than of the oxidation of $NH_4^+$ to $NH_2OH$. In fact, the latter transformation has not as yet been accomplished with a cell-free system. Anderson (2) reported that the reaction was endergonic and initially suggested that $NH_4^+$ oxidation required an expenditure of high-energy phosphate. He subsequently postulated (3) activation of $NH_4^+$ by oxygen in the form of peroxide ($H_2O_2$). Peroxide may be generated by the oxidation of a reduced carrier (flavine) in the electron transport system that links $NH_2OH$-cytochrome *c* reductase and atmospheric oxygen. Of particular interest in this regard is Bonazzi's publication (6), which described the chemical oxidation of $NH_4^+$ to $NO_2^-$ by $H_2O_2$ and suggested that *Nitrosomonas* may oxidize $NH_4^+$ by means of a catalytic system involving $H_2O_2$.

Cells of *Nitrosomonas* contain both catalase (10) and peroxidase (27), but peroxidase is the more likely to be physiologically involved because it uses a wider range of inorganic and organic electron donors, and it is able to function in certain oxygenase reactions (30). This paper describes the purification and properties of peroxidase from *N. europaea* and presents the results of experiments designed to determine whether the enzyme acts to catalyze the oxidation of $NH_4^+$ to $NO_2^-$.

## MATERIALS AND METHODS

*Cultivation and collection of cells. N. europaea* was cultivated in 1.0-liter Erlenmeyer flasks that contained 500 ml of an inorganic salts medium described previously (20). Each flask was inoculated with 5 ml of a pure culture of the bacterium in the log phase of development and incubated for 7 days on a rotary shaker at 28 C. Cells were harvested with a Sharples continuous-flow centrifuge, operating at 25,000 rev/min with an input rate of 200 ml of culture medium per min. Pellets were washed with 0.03 M phosphate buffer at *p*H 7 until spot tests indicated that they were free of residual $NH_4^+$ and $NO_2^-$. All centrifugation and washing procedures were performed in a cold

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room at 4 C with precooled equipment and reagents. The average yield of cells was approximately 30 mg (dry weight) per liter of medium, and the final product was a brick-red paste.

*Analyses.* $NH_4^+$, $NO_2^-$, and nitrate ($NO_3^-$) were determined with Nessler's and the Griess-Ilsovay reagents (28), and $NH_2OH$ was estimated by the method of Csaky (8). A Klett-Summerson colorimeter (Klett Manufacturing Co., New York, N.Y.) equipped with a blue filter (no. 42) was used in this investigation. $H_2O_2$ concentration was measured volumetrically by titration with thiosulfate as described by Herbert (14).

Protein levels were determined spectrophotometrically by absorption at 280 and 260 mμ and also by means of the Folin-Ciocalteau phenol reagent as described by Layne (16). Organic nitrogen was estimated by use of the micro-Kjeldahl method outlined by Pelczar, Hensen, and Konetzka (26).

All spectrophotometric analyses were conducted with a Beckman DU instrument fitted with a constant-temperature water jacket at 30 C.

*Enzyme activity.* Peroxidase was assayed as suggested by Luck (22). The reactants and the order of their addition to a cuvette were as follows: enzyme, 2.0 ml; 67 mM sodium phosphate at *p*H 7, 1.0 ml; 30 mM $H_2O_2$, 0.1 ml; and 1.0% *p*-phenylenediamine, 0.1 ml. The solutions of *p*-phenylenediamine and $H_2O_2$ were prepared daily. When the reaction mixture was complete, the cuvette was inverted twice and then placed in a spectrophotometer at 30 C. Changes in absorbance ($\Delta A$) at 485 mμ were noted at 15-sec intervals for 3 min, and the peroxidatic activity per ml of the preparation assayed ($P$) was expressed as $\Delta A$/min. Reaction mixtures that lacked enzyme or $H_2O_2$ were included as controls, and lyophilized horseradish peroxidase purchased from Worthington Biochemical Corp. (Freehold, N.J.) was used as a standard.

*Peroxidase purification procedure.* Cells, stored in a frozen state until sufficient material was accumulated for fractionation and enzyme purification, were thawed and disrupted by sonic treatment (M. S. E. Ultrasonicator, Mullard Equipment Ltd., England) in the cold for 60 min. Unbroken cells and debris were concentrated by centrifugation and discarded. The opalescent supernatant liquid was employed as a source of enzyme. Nucleic acids were removed by precipitation with streptomycin sulfate (1.0%). Finely ground $(NH_4)_2SO_4$ was added to the supernatant fluid with constant stirring at 4 C, and protein that was insoluble at salt concentrations of 60 to 75% was recovered and submitted to gel filtration on Sephadex G-100 with 0.01 M phosphate buffer at *p*H 7 as eluent. Gel filtration served simultaneously to desalt and fractionate the product of $(NH_4)_2SO_4$ precipitation. Active fractions of the effluent were resubmitted to gel filtration and then combined and concentrated. The purified peroxidase produced by this procedure was characterized by conventional methods and tested as a catalyst for the oxidation of $NH_4^+$ to $NH_2OH$ by $H_2O_2$.

*Molecular weight by gel filtration.* The molecular weight of the purified peroxidase was estimated by the method of Andrews (4). Sephadex G-100 with a particle size of 40 to 120 μ purchased from Pharmacia Fine Chemicals (Piscataway, N.J.) was equilibrated with 0.01 M phosphate buffer at *p*H 7. The protein standards used to calibrate the column (85 × 2.5 cm) were selected to give a wide range of molecular weight and were of the highest purity commercially available. The exclusion volume was determined by the appearance of Blue Dextran 2000 and the internal volume with glucose. Chromatography was performed at 4 C with a hydrostatic head of 15 cm, and effluents were collected in 3.5-ml fractions.

## RESULTS AND DISCUSSION

Extracts of *Nitrosomonas* obtained by sonic treatment of cells contained both catalase and peroxidase. Since catalase is approximately six times the molecular size of peroxidase, they were easily separated by gel filtration on Sephadex G-100. A 1,500-fold increase in specific activity of the peroxidase was ultimately achieved by the purification procedure.

The results of an analysis by the method of Andrews (4) to determine the molecular weight (MW) of the peroxidase purified from *Nitrosomonas* are summarized in Fig. 1. Agreement was excellent between the elution volumes obtained with reference proteins by Andrews (4) and those in this study. *Nitrosomonas* peroxidase obtained by gel filtration of the ammonium sulfate fraction emerged as one major peak when rechromatographed on Sephadex G-100. Comparison of the elution patterns for activity and protein revealed that each coincided with heme absorbance at 410 mμ. The elution volume for this bacterial peroxidase was compatible with a molecular weight of approximately 53,000. This

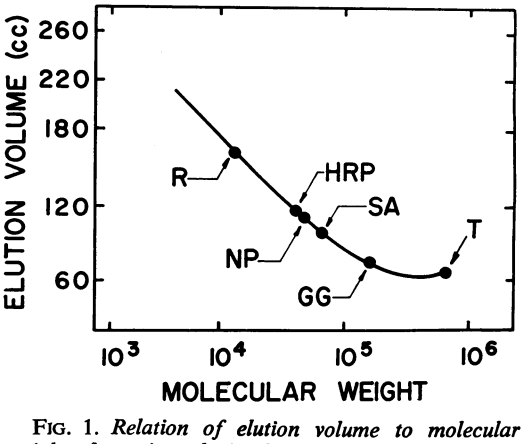


FIG. 1. *Relation of elution volume to molecular weight of proteins submitted to gel filtration on Sephadex G-100. Symbols: R, ribonuclease; HRP, horseradish peroxidase; NP, Nitrosomonas peroxidase; SA, serum albumin; GG, gamma globulin; T. thyroglobulin.*

value is greater than that which is usually assigned to horseradish peroxidase (40,000), but it compares favorably with the molecular weight of 60,000 reported for crystalline peroxidase obtained from yeast (1).

The absorption spectrum of purified *Nitrosomonas* peroxidase (Fig. 2) revealed three distinct maxima at 410, 524, and 558 mμ. The addition of substrate ($H_2O_2$) caused a shift of the 410-mμ peak to 417 mμ, indicating complex formation between the enzyme and its primary substrate. The spectrum of the *Nitrosomonas* enzyme is not identical with any described by Paul (25) or Saunders et al. (30), but there is correspondence, in the Soret region, with absorption by the peroxidase of yeast. There is little qualitative difference in the spectra of heme proteins, and quantitative differences are not great enough to permit the identification of an enzyme to be based on spectral characteristics only.

Tests of the influence of temperature on enzyme stability and activity demonstrated that denaturation was initiated after 30 min at 40 C and established that 44 C was the temperature optimum.

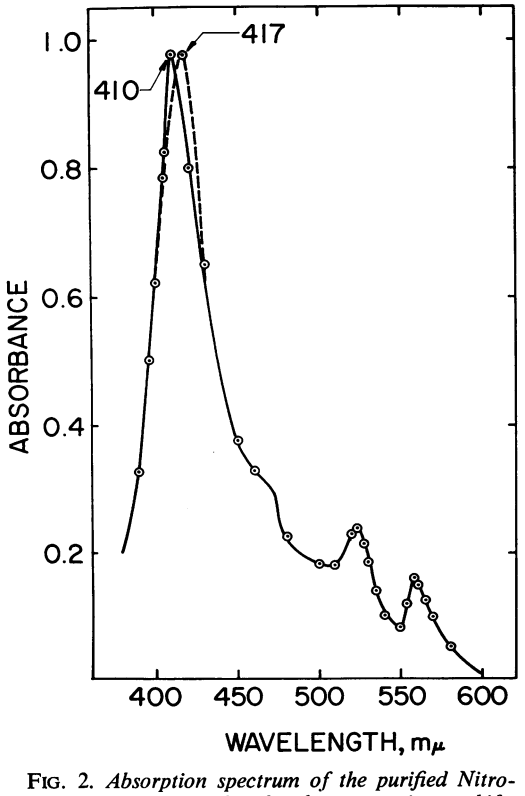


FIG. 2. *Absorption spectrum of the purified Nitrosomonas peroxidase. The absorbance maximum shifts to 417 mμ upon addition of hydrogen peroxide.*

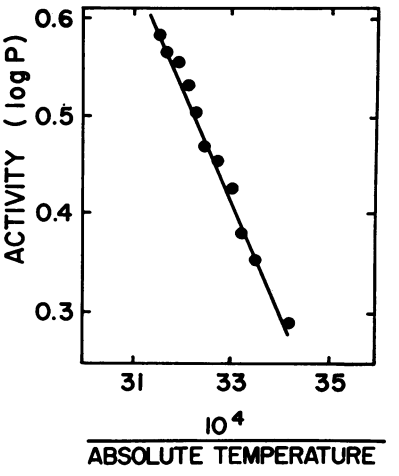


FIG. 3. *Influence of temperature on activity of Nitrosomonas peroxidase.*

The energy of activation of the peroxidase reaction was calculated as 5,850 cal/mole from the data summarized in Fig. 3. The enzyme was less stable at acid than at alkaline *p*H and was not influenced adversely by storage at 30 C for 30 min in buffer solutions of *p*H 6 to 8. The relation of activity to *p*H was described by a bell-shaped curve that indicated an optimum at *p*H 7.5. The effects of temperature on the stability and activity of peroxidase do not appear to have been described previously. Likewise, no report of an energy of activation was located in the literature. Present results are consistent with reports by others (22) that peroxidase acts over a wide *p*H range and has an optimum that is generally close to neutrality but varies with the nature of the hydrogen donor employed in the assay procedure.

Peroxidase activation and inhibition have been considered by various investigators. Whitaker and Tappel (31) described salt-induced increases in peroxidase activity, and enzyme activation by NaCl, KCl, and $Na_2SO_4$ was noted in the present studies. $Na_2SO_4$ had the greatest effect. Activation was a function of salt concentration, and, at a level of 2 M, reaction rates were almost doubled. The results listed in Table 1 agree generally with those of other investigators who have studied peroxidase inhibition (5, 9, 30). The enzyme was most sensitive to sulfide (complete inhibition at $10^{-4}$ M), and inhibition by sulfide and cyanide was reversible. Fluoride had little or no effect on the *Nitrosomonas* enzyme (20% inhibition at 1.0 M), although it poisoned horseradish peroxidase at levels of $10^{-3}$ M or greater (5). A relative resistance of peroxidase to 2,4-dinitrophenol is one of the characteristics that differentiates it from

TABLE 1. *Inhibitors of Nitrosomonas peroxidase*

| Compound | Concn (M) | Inhibition[a] (%) |
|---|---|---|
| Sodium azide | $3 \times 10^{-1}$ | 100 |
| | $3 \times 10^{-2}$ | 50 |
| Sodium cyanide | $3 \times 10^{-2}$ | 100 |
| | $3 \times 10^{-4}$ | 30 |
| Sodium sulfide | $1 \times 10^{-4}$ | 100 |
| | $1 \times 10^{-5}$ | 20 |
| Hydroxylamine-HCl | $3 \times 10^{-2}$ | 100 |
| | $3 \times 10^{-4}$ | 50 |
| 2,4-Dinitrophenol | $3 \times 10^{-2}$ | 100 |
| | $3 \times 10^{-3}$ | 50 |
| Ethyl xanthate | $1 \times 10^{-3}$ | 100 |

[a] Enzyme was exposed to inhibitor for 30 min at 28 C and then assayed at *p*H 7.0.

catalase (13). Apparently, *Nitrosomonas* peroxidase differs from *Pseudomonas* cytochrome *c* peroxidase, since the former but not the latter is sensitive to ethyl xanthate (19). Thiourea prevents the oxidation of $NH_4^+$ to $NH_2OH$ by growing cultures of *N. europaea* (17), but it did not influence activity of the purified peroxidase. Much has been written of enzyme activation and inhibition; however, "it is evident that a comprehensive experimental study of the effects of precise factors on the activities of purified peroxidases is very much needed" (30).

Substrate concentration is a major determinant of the velocity of enzyme reactions, but peroxidase requires two substrates: a primary substrate that must be a peroxide (hydrogen peroxide, methyl- or ethylhydroperoxide) and a secondary substrate that can be any one of a great variety of hydrogen donors (25). The kinetics of the peroxidase catalyzed reactions are extremely complex, but the Michaelis-Menton constant ($K_m$) for the system continues to be measured as a function of concentration of the primary substrate (9, 25). To obtain a $K_m$ value for the *Nitrosomonas* peroxidase-$H_2O_2$-phenylenediamine system, tests were performed with 0.5 $\mu$g of enzyme per ml in *p*H 7 phosphate buffer at 40 C, and $H_2O_2$ was added at levels ranging from $0.5 \times 10^{-5}$ to $10^{-4}$ M. The concentration of primary substrate required for the reaction to proceed at half maximal velocity ($K_m$) was estimated at $7 \times 10^{-6}$ M by the method of Lineweaver and Burk (21). The magnitude of the *Nitrosomonas* constant is comparable to the $K_m$ values of $5 \times 10^{-7}$ and $5 \times 10^{-6}$ M reported for horseradish (24) and *Pseudomonas* (23) peroxidase, respectively. *Nitrosomonas* peroxidase was able to use a variety of hydrogen donors, including reduced cytochrome *c*, pyrogallol, and *p*-phenylenediamine, but of particular interest was the possibility that $NH_4^+$ may function in this capacity and be enzymatically transformed to $NH_2OH$ or $NO_2^-$. If the peroxidation of $NH_4^+$ by this mechanism was coupled to the generation of high-energy phosphate or reducing power, it would be physiologically significant and a vital step in the nitrification process.

Furthermore, Bonazzi (6) reported that $NO_2^-$ was formed in a solution that contained $(NH_4)_2SO_4$ (25 ml of a 0.2% solution), $Na_2CO_3$ (1.0 ml of a 5% solution), and $H_2O_2$ (5 ml of a 3% solution) and was incubated at 30 C for 60 min. This chemical oxidation of $NH_4^+$ to $NO_2^-$ by $H_2O_2$ was confirmed, and a series of tests were then performed to measure the effects on the reaction of *p*H, $H_2O_2$ concentration, and $(NH_4)_2SO_4$ concentration. The conditions found most suitable for the $NH_4^+$ to $NO_2^-$ transformation were $H_2O_2$ and $(NH_4)_2SO_4$ levels of 0.21 and 0.11%, respectively, in a solution at *p*H 8.5. It was possible to substitute $NH_2OH$ for $(NH_4)_2SO_4$ in the system, but attempts made at 5-min intervals for 60 min to detect $NH_2OH$ as an intermediate in the formation of $NO_2^-$ from $NH_4^+$ were unsuccessful. The chemical oxidation of $NH_4^+$ to $NO_2^-$ by $H_2O_2$ was rapid and complete, and it did not proceed to $NO_3^-$. Neither the rate nor the extent of the reaction was influenced by addition of *Nitrosomonas* or horseradish peroxidase to the above system. Although environmental conditions favorable for the chemical oxidation of $NH_4^+$ to $NO_2^-$ are similar to those described as optimal for growth of *Nitrosomonas*, the transformation could be of no benefit to the organism. In fact, $H_2O_2$ will compete with *Nitrosomonas* for substrate rather than transform $NH_4^+$ to $NH_2OH$ or some other compound that is available to the organism for oxidation to $NO_2^-$. The results of these studies provided no support for the suggestion of Anderson (3) that $NH_4^+$ is activated by $H_2O_2$, and no physiological role could be demonstrated for peroxidase in the *Nitrosomonas* system for oxidizing $NH_4^+$ to $NO_2^-$.

Chance (7) has emphasized that two problems arise in any consideration of the role of peroxidase. The first is the source of peroxide, and the second is the nature or identity of the donor molecule. The most commonly recognized sources of $H_2O_2$ are oxidase systems, especially the flavine enzymes. Flavine was detected in particulates from *Nitrosomonas*, and flavoprotein is included in the electron transport system suggested by Falcone et al. (11) as functioning in the bacterium during oxidation of $NH_2OH$ to $NO_2^-$. However, the nature of the donor molecule is not known. Present studies indicate that it is probably

not $NH_4^+$, but many other possibilities exist. Peroxidases, in general, can oxidize a number of metabolically significant compounds, including cytochrome *c*, ascorbic acid, tryptophan, long-chain fatty acids, glutathione, phenylalanine, tyrosine, and others (30). Peroxidase may perform a peripheral rather than a vital role, but, depending on the identity of the physiological donor, the enzyme in *Nitrosomonas* may catalyze an oxidation step in some metabolic sequence, or, possibly, act on a substrate such as cytochrome *c* to form part of an alternate respiratory chain.

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